Master's Thesis

STIM2β is upregulated by insulin signaling and involved in adipogenesis

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2015

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A thesis
submitted to the Graduate School of UNIST
in partial fulfillment of the
requirements for the degree of
Master of Science

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7. 15. 2015

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7. 15. 2015

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Abstract

Calcium ion play an important role in lipid formation and adipogenesis. The STIM1 and STIM2 are endoplasmic reticulum Ca2+ sensors which activate and regulate Orai, store operated calcium channel. I am interested in stim2β which is a splicing variant of STIM2 containing cassette exon 9 converting STIM2 from an activator to an inhibitor. To know the role of stim2β in adipogenesis, CRISPR system is applied. Through this process, I made several Stim2β knock out cell lines in NIH 3T3-L1 called as L1-S2β-KO #5, #13, and #14. As a result, Stim2β is not involved in proliferation through MTT assay but involved in early differentiation through Oil red O staining. In addition, Stim2β knock out cell lines show faster adipogenesis than wild type by observing expression difference of PPARγ2, aP2 which is differentiation positive markers. Also, Stim2β is affected by insulin signaling during adipogenesis. Generally, SOCE components are affected by insulin signaling, too. Especially, semi quantitative PCR and quantitative RT-PCR reveal that Stim2 isoforms have oscillation pattern after 24 hours of insulin treatment. Therefore, I suggest that regulation of STIM2β through insulin signaling can be potential therapeutic approach of obesity and related metabolic disorder.

KEYWORDS : adipogenesis, STIM, Stim2α, Stim2β, insulin, CRISPR, NIH 3T3-L1, SOCE components, differentiation, proliferation.

ABBREVIATIONES USED

3T3-L1 : murine preadipocytic line derived from National Institute of Health (NIH)

3T3 : 3-day transfer, inoculum 3 x 10^5 cells

aP2 : adipocyte Protein 2

CRISPR : Clustered Regularly Interspaced Short Palindromic Repeats

crRNA : CRISPR RNAs

DEX : dexamethasone

GAPDH : Glyceraldehyde 3-phosphate dehydrogenase

IBMX : 3-isobutyl-1-methylxanthine

L1-S2β-KO : Stim2β knock out cell line in NIH 3T3-L1

PPARγ2 : peroxisome-proliferator-activated receptor gamma 2

qRT-PCR, quantitative reverse-transcription PCR

SOCE : Store Operated Calcium Entry

STIM : Stromal interaction molecule

tracrRNA : trans-activating crRNA

CONTENTS

Introduction

Recently, Obesity is a major public health interest, with its broad cause of increase in diabetes, hypertension and cardiovascular diseases (Kopelman 2000) . Especially, if intake of food go over the energy expenditure chronically, most excess energy accumulated as triacylglycerols and cause increase of fat tissue (Gregoire, Smas et al. 1998). Thus, the regulation of adipogenesis is one of the most important medical attentions because obesity is related with hypertrophy of adipocytes and increases of adipogenesis.

Intracellular calcium level is important for adipogenesis. Previous study shows that an increase of intracellular calcium ion concentration in adipocytes often brings obesity development (Draznin, Sussman et al. 1988) . Intracellular calcium ion level oscillations are related not only cell proliferation and differentiation, but also the regulating control of calcium concentration (Dolmetsch, Xu et al. 1998). The oscillating frequency of calcium ion level has been known to regulate gene expression in various cell types (Dolmetsch, Xu et al. 1998). However, the role of intracellular calcium ion is not well known in human pre-adipocytes. Thus I want to figure out the relationship between the adipogenesis and intracellular calcium oscillation which is regulated by Store Operated Calcium Entry (SOCE)

SOCE which mediates cytosolic calcium signals regulates various cellular functions such as transcription, differentiation, secretion, motility and apoptosis. The most well-established SOC channel is the Calcium Release-Activated Calcium (CRAC) Channel. SOCE is occurred by the calcium store depletion in ER, generally after activation of cell membrane receptors. The key mediators of SOCE are Stromal Interaction Molecule (STIM) family (Liou, Kim et al. 2005) and the Orai calcium channels (Feske, Gwack et al. 2006). Binding of STIM with Orai opens the channel through a nonlinear process which is greatly sensitive to binding stoichiometry (Hoover and Lewis 2011).

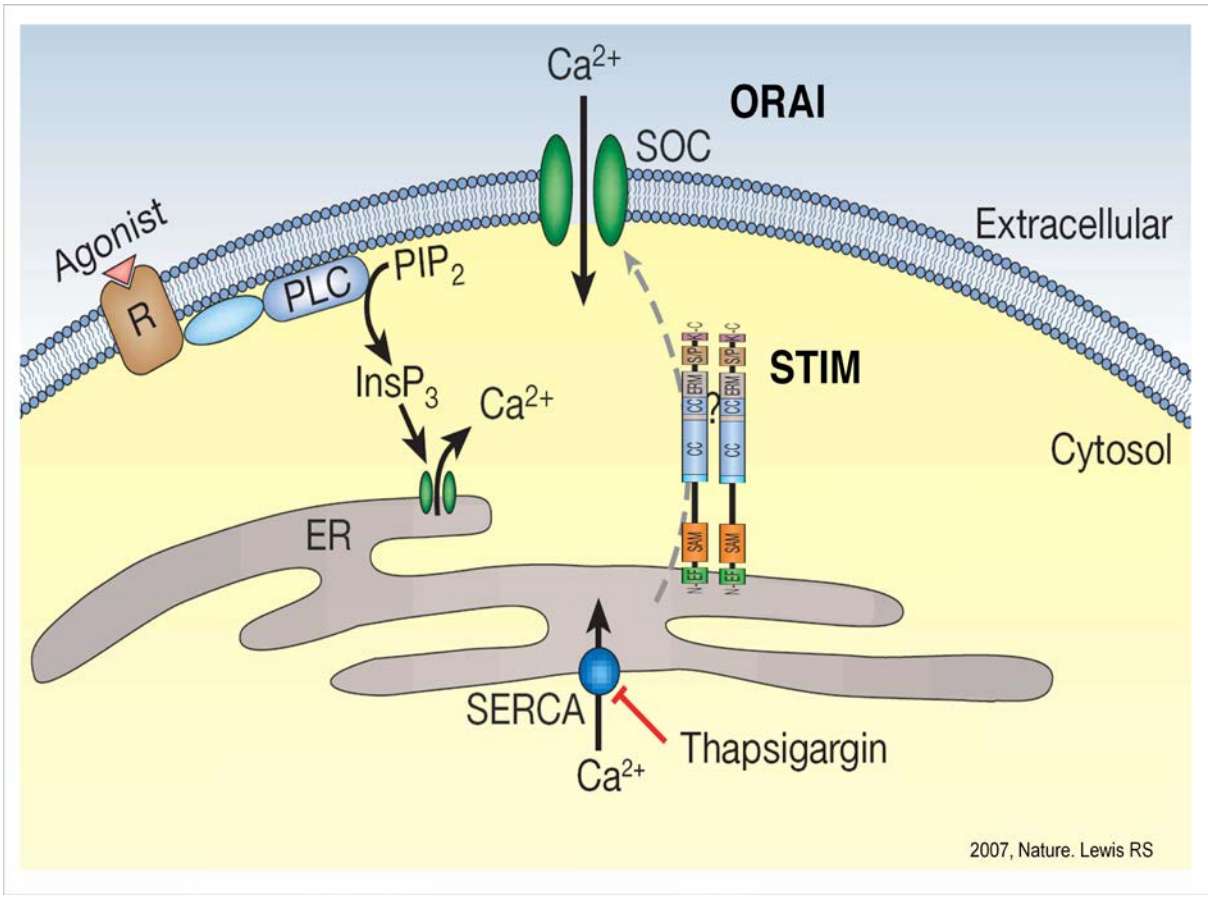
The Role of SOC components and its subtypes are still being discovered. Recently research group show that stim2β is an inhibitor of stim1 (Rana, Yen et al. 2015). STIM2β is alternative splicing variants of STIM2. This splicing variant is highly conserved. STIM2β has exon 9 which has 24 base pairs while STIM2α which is another splicing variant of STIM2 does not have one (Rana, Yen et al. 2015). Absence or presence of 24 base pairs make the functional difference of STIM2. Thus, it is

important to know the role of STIM2β in adipogenesis.

Adipogenesis means the differentiation of pre-adipocyte to adipocyte. This adipocyte differentiation involves various factors such as cAMP, insulin and glucocorticoids (Rosen and Spiegelman 2000) . Therefore, the best representative cellular model of adipogenesis is 3T3-L1 cells which are fibroblast line. 3T3-L1 cells are treated with 3-isobytyl-1-methylxanthine (IBMX) which is phosphodiesterase inhibitor, insulin which increases cAMP levels and Dexamethasone (DEX) which is synthetic glucocorticoid. 3T3-L1 cell line accumulates lipid droplets and change to the synchrously morphology of mature adipocyte (MacDougald and Lane 1995). Growth arrest, mitotic clonal expansion, and differentiation are the major events of adipogenesis. This differentiation process can be characterized by the chronological change of protein markers in early, intermediate, and late state and of accumulation of triglyceride.

To establish the role of STIM2β in adipogenesis, I generated the STIM2β specific knock out cell line with cutting edge genomic DNA engineering system. Several research groups have published the use of CRISPR system for genomic DNA editing in various species (MacDougald and Lane 1995). CRISPR system is originated from the type 2 adaptive immune system called CRISPR/Cas9 in prokaryotic cell. CRISPR system is operated by targeting 20 nucleotide sequence upstream of 5`-NGG Protospacer Adjacent Motif (PAM) sequence. sgRNA which is consist of crRNA and tracrRNA recognize the specific target sequence and Cas9 nuclease cut the PAM sequence in target site. The site-specific breaks which occurs in both double strands are repaired by Non-homologous end-joining (NHEJ) with random editing (Barnes 2001) or by homologous recombination. The highly effective and simple principle of CRISPR system boosts us to validate its function for gene engineering of 3T3-L1 cell which is pre-adipocyte.

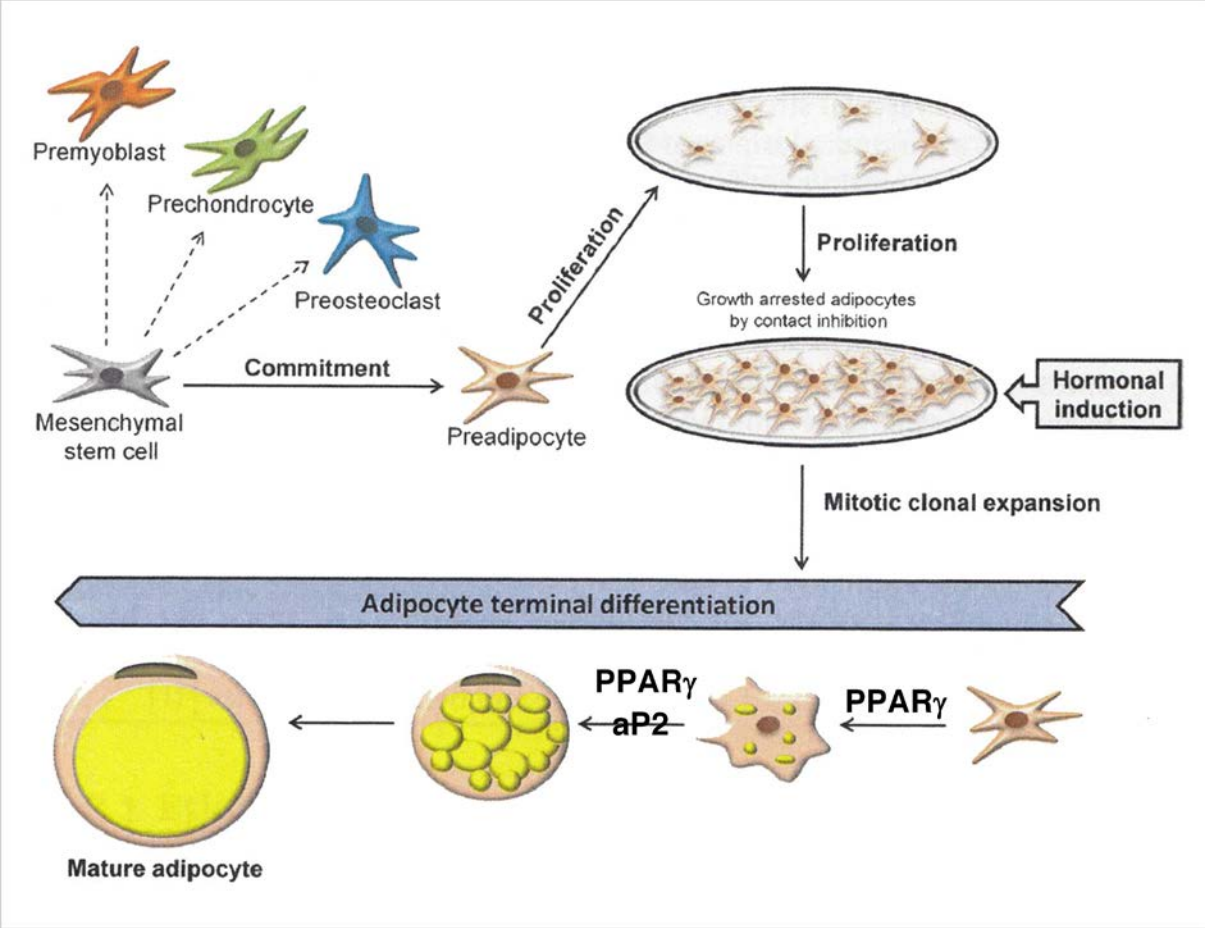
Therefore, I demonstrated that STIM2β is involved in differentiation not proliferation. Also, STIM2β knock out cell line shows faster adipogenesis than wild type. Furthermore, the gene expression of STIM2 is regulated by insulin signaling. Thus, this research suggests that imbalance of calcium ion concentration in cytosol is regulated by STIM2β which is regulated by insulin signaling and this modulation is important for adipogenesis.



(Lewis 2007)

Introduction figure 1. SOCE components

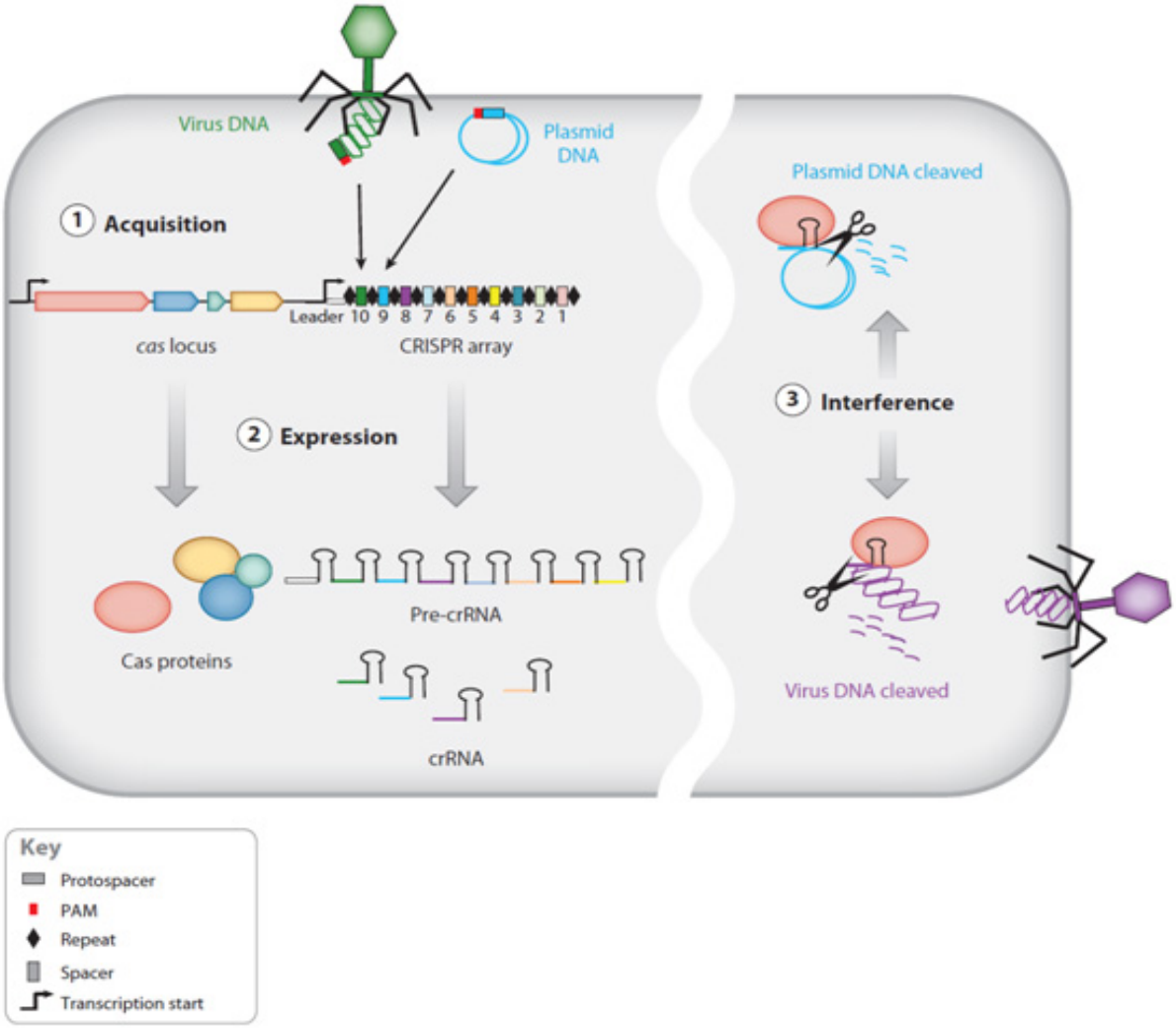
SOCE consists of Orai which is plasma membrane calcium channel pore forming protein and STIM which is Ca2+ channel regulator protein by sensing calcium ion concentration in ER. If some agonists bind to their receptor, Phospholipase C (PLC) is activated. Activated PLC divides phophatityl-inositol-4,5-bisphosphate (PIP2) into Inositol-1,4,5-triphosphate (IP3) and diacylglycerol (DAG). IP3 stimulates Ca2+ leak from ER by targeting IP3 receptor. When Ca2+ concentration is decreased in ER, EF hand which is part of STIM loses Ca2+ and stim induces a conformational change and moves into ER-PM Junction. Therefore, STIM binds to Orai and then Ca2+ come into cytosol. This Ca2+ influx is called SOCE which is Store Operated Calcium Entry.



(Adapted from Romao and Guan, 2015. microRNA in Regenerative Medicine)

Introduction figure 2. Adipogenesis procedure

Bone marrow derived mesenchymal stem cell can be committed to pre-adipocyte. Pre-adipocyte proliferates until becoming confluent state. After confluent state, pre-adipocyte undergoes mitotic clonal expansion. Then, differentiation signal induces differentiation of pre-adipocyte into mature adipocyte. Mature adipocyte has distinctive morphology such as accumulation of lipid inside of cell. This process called adipogenesis. During adipogenesis, PPARγ2 and aP2 proteins are increased. Therefore, these two proteins have been used as differentiation marker for adipocyte differentiation.



(Bhaya, Davison et al. 2011)

Introduction figure 3. CRISPR mechanism

The ability to specifically and sustainably modify expression endogenous gene by targeting genome editing is a largely effective genetics tool. Recently, genomic DNA engineering has developed surprisingly with the principle of bacterial CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) systems and its application in mammalian cells.

1. Acquisition through unity of external DNA-derived spacers into CRISPR locus
2. Expression of CRISPR RNAs (crRNAs) containing single spacer-repeat unit
3. Interference of plasmid or viral invasion through cleavage of targeting sequence

Materials and methods

1. Cell culture

NIH 3T3-L1 cells (American Type Culture Collection Rockville, MD) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum (BCS) heated at 65℃ for 15 minutes, at 37 ℃ in a 5% CO2 humidified atmosphere (Burton, Guan et al. 2002). For experiments, 3T3-L1 cells were seeded at a density of $1x10^5$ cells/ cm^2 and let them grow to confluence. Two days post-confluent state, cells were cultured with growth media(GM) medium consisting of DMEM supplemented with 10%(v/v) BCS (Gibco) (Burton, Nagarajan et al. 2004). Cells were stimulated for differentiation by differentiation media consisting of DMEM supplemented with 10%(v/v) FBS (Gibco), 3-isobutyl-1-methylxanthine (M; 0.5mM), dexamethasone (D; 1uM) and insulin (I; 10ug/ml) (Sigma, St.Louis, MO) (Richon et al., 1997).

2. Oil red O staining

After 3days and 5days of adipogenic differentiation from the point of Inducing MDI after staying 2 days in confluent state (day 0), differentiated cells were fixed in 4% paraformaldehyde for 2 hours and then stained with 2.1mg/ml Oil Red O(Sigma) in 60% isopropanol for 40 minutes(Graham, Black et al. 2009). These stained cells were washed with distilled water three times. Images of Cells were obtained and Oil Red O staining was eluted with 100% isopropanol and quantified by obtaining the optical density (OD) at 490 nm(Park and Sung 2015). Stained cell images were obtained by fluorescence inverted microscopy IX71, Olympus in UNIST Central Research Facilities

3. semi quantitative PCR

Semi quantitative RT–PCR experiments for differentiation were performed to establish our approaches. Differentiated cell were extracted every 12 hours or 24 hours with Ribo-Ex reagent to isolate total RNA according to the manufacturer's instructions. The cDNA was synthesized from total RNA using Toyobo cDNA Synthesis Kit. And PCR was performed by using cDNA as the template in a reaction mixture containing targeted primer pair of each cDNA (Hung, Chang et al. 2004). Primer sequence is designed according to the attached sequences (Table 1, Designed primer for RT-PCR).

The reaction mixture was incubated at 94℃ for 3 min initially, followed by 35-50 cycles of denaturation at 94 ℃ for 30 sec, annealing at 55℃ for 30 sec, and extension at 68 ℃ for 30 sec(Hung, Chang et al. 2004). The PCR products were separated by electrophoresis in 1.5% Etbr agarose gel and photographed by Bio-Rad Gel Doc.

4. Quantitative real-time PCR

RNA extraction was performed by using Ribo-Ex following the instructions of manufacturer. The RNA was reverse-transcribed to cDNA by using Toyobo cDNA Synthesis Kit following the manufacturer's instructions. Synthesized products were detected by measuring fluorescence (Yang, Yin et al. 2014). Quantification of cDNA for each differentiation gene was established by real-time PCR using a LightCycler 480Ⅱ (Roche Life Science). The cycle threshold values (Ct values) were obtained by LightCycler 480 Software 1.5. The expression level of each differentiation gene is normalized with the amount relative to 36B4. (Table 1, Designed primer for qRT-PCR)

5. Cell viability (MTT assay)

Cells were seeded in 96 well in various population of cell. The cultured cells were incubated with treatment of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution in media for 3 hours at 37℃ (Park and Sung 2015). Then solution added media is removed. And the formazan crystals were dissolved with dimethyl sulfoxide (DMSO). After suspense the solution, absorbance was measured at 540 nm by using a spectrophotometer.

Table 1. PCR Primers for RT-PCR and qRT-PCR

Gene	Product size (bp)		Sequence (sense and antisense)
mOrai1	451	F	5' – CCTGCATCCTGCCCAACATCGAGGC – 3'
		R	5' – TCCCCTCTGTGGTCCAGCTGGTCC – 3'
mOrai2	387	F	5' – GGCCATGGTGGAGGTGCAGCTGGAG – 3'
		R	5' – ACCATGATGATGGTGGACACCAG – 3'
mOrai3	375	F	5' – TGGGTCAAGTTTGTGCCCATTGG – 3'
		R	5' – CACAGCCTGCAGCTCCCCCTGC – 3'
mStim1	374	F	5' – TGGCCTGGGATCTCAGAGGG – 3'
		R	5' – TGTCCCCAACTGGAGATGGTGTG – 3'
mStim2	481	F	5' – AAGATCTGTGGCTTTCAGATAGC – 3'
		R	5' – AGTCACATTCTGAAGCTGTGTCTGG – 3'
mStim2α	187	F	5' – GCTAGCCATCGCTAAGGACGAGGCAG – 3'
		R	5' – AGCTATCTGAAAGCCACAGATCTTCTC – 3'
mStim2β	188	F	5' – TCGCTGCCTCCTATCTCCTGCAGG – 3'
		R	5' – AGCTATCTGAAAGCCACAGATCTTCTC – 3'
PPARγ2	112	F	5' – TCTGGGAGATTCTCCTGTTGA – 3'
		R	5' – GGTGGGCCAGAATGGCATCT – 3'
aP2	113	F	5' – GAATTCGATGAAATCACCGCA – 3'
		R	5' – CTCTTTATTGTGGTCGACTTTCCA – 3'
actin	138	F	5' – GATCTGGCACCACACCTTCT – 3'
		R	5' – GGGGTGTTGAAGGTCTCAAA – 3'
GAPDH	176	F	5' – TGCACCACCAACTGCTTAG – 3'
		R	5' – GATGCAGGGATGATGTTC – 3'
36B4	252	F	5' – AATCTCCAGAGGCACCATTG – 3'
		R	5' – CCGATCTGCAGACACACACT – 3'

Table2. Composition of differentiation media

Materials	Components
MDI	Isobutyl methyl xanthine (0.5mM)
	Dexamethaxon (1uM)
	Insulin (10ug/ml)
Growth Media (GM)	10% BCS DMEM
Differentiation Media (DM)	10% FBS DMEM

Table3. Number of nucleotide deletion mutation in L1-S2β-KO #13 genomic DNA analyzed by sequencing.

Deletion phenotype	Number of colony
WT	3 (14.3%)
△2NT	6 (28.6%)
△3NT（TAT）	3 (14.3%)
△3NT（TCT）	3 (14.3%)
△7NT	2 (9.5%)
△9NT	4 (19%)

Table4. Number of nucleotide deletion mutation in L1-S2β-KO #14 genomic DNA analyzed by sequencing.

Deletion phenotype	Number of colony
3NT Δ	8 (23.5%)
5NT Δ	6 (17.5%)
7NT Δ	6 (17.5%)
9NT Δ	2 (5.5%)
21NT Δ	5 (14.5%)
198NT Δ	7 (20.5%)

Table5. Number of nucleotide deletion mutation in L1-S2β-KO #5 genomic DNA analyzed by sequencing.

Deletion phenotype	Number of colony
2NT Δ	17 (94.5%)
455NT Δ	1 (5.5%)

Table6. Spectrophometric quantitation of Oil Red O elution with DMSO at day 3, 5 and 7 post MDI treatment of 3T3 L1 wild type, L1-S2β-KO #13 and L1-S2β-KO #14.

DAY 3			DAY 5			DAY 7		
3T3-L1	S2β–KO #13	S2β–KO #14	3T3-L1	S2β–KO #13	S2β–KO #14	3T3-L1	S2β–KO #13	S2β–KO #14
0.9642	1.7896	1.4563	1.6143	2.4955	2.1466	2.1813	2.9992	2.8349
0.9689	1.7092	1.4346	1.6354	2.4824	2.1982	2.1638	3.0401	2.8331
0.9489	1.7037	1.4506	1.6329	2.4536	2.1495	2.1315	2.9545	2.8316
0.9279	1.6763	1.386	1.6098	2.4672	2.0283	2.1015	3.0089	2.8155

Chapter 1.

Results

Part 1. STIM2β is involved in adipogenesis.

1. Differentiation of NIH 3T3-L1 cells into adipocytes.

The NIH 3T3-L1 pre-adipocyte cell differentiates effectively into morphologically different, triglyceride burdened adipocytes over 7 days of differentiation period after cells in confluent state being induced with a differentiation cocktail containing 3-isobutyl-1-methylxanthine(IBMX), dexamethasone (DEX) and insulin (MDI) (Cowherd, Lyle et al. 1999).

I confirmed that differentiation of NIH 3T3-L1 was well induced (Fig. 1a, top panel). In addition, I confirmed the formation of lipid droplet through Oil red O staining (Fig. 1a, down panel) (Serlachius and Andersson 2004). To check differentiation which is increase of adipogenesis positive maker PPARγ2 and aP2, I did RT-PCR experiment (Fig 1b) (Pei, Yao et al. 2011). mRNA level of PPARγ2 and aP2 was gradually increased during adipogenesis. The result of qRT-PCR are consistent with that of RT-PCR (Fig 1c, d). Quantified results also are in agreement with this experiment (Fig 1e, f).

2. Expression of Stim and Orai isoforms during adipogenesis of 3T3 L1 cells.

The mRNA level of SOCE components were detected by RT-PCR using the primers (Table1) in NIH 3T3-L1 cells (Fig 2a). The RT-PCR result showed that NIH 3T3-L1 expresses Orai1, Orai3, Stim1 and Stim2 (Graham, Black et al. 2009). Also they may be involved in differentiation since their expression is changed during adipogenesis. Thus, to confirm the change of mRNA level of Stim2α and Stim2β which is Stim2 splicing variant during adipogenesis, I measured the mRNA expression difference with RT-PCR (Fig 2b). When I treat MDI at day0 and treat insulin at day2, mRNA level of Stim2α and Stim2β is increased. I confirmed that adipogenesis is going well in this experiment by observing Oil red O staining experiment (Fig 2c) (Graham, Black et al. 2009)

3. Generation of stim2β knock-out L1 cell using CRISPR system.

We designed this primer to detect Stim2 isoforms. To confirm expression of Stim2β mRNA level,

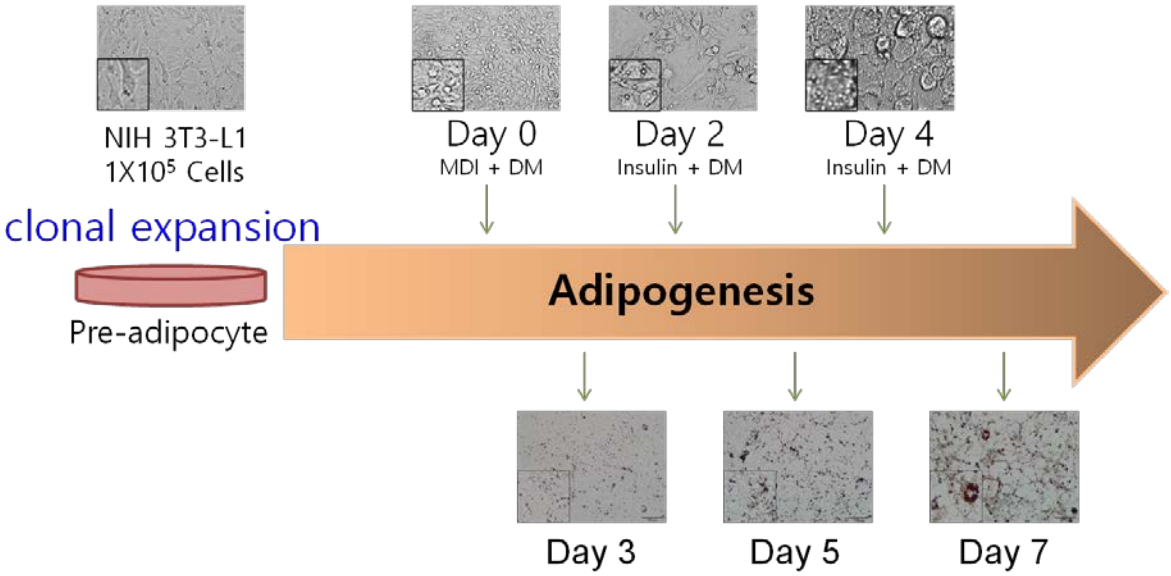
the primer 1 bind to both the end of exon8 and the beginning of exon10 because Stim2β doesn`t contain exon 9. But stim2β contains exon9. To confirm expression level of Stim2β mRNA level, the primer 2 bind exon9. Primer 3 is used for common (Fig 3a). CRISPR system consists of Cas9 nuclease, tracrRNA, crRNA and PAM sequence (Miederer, Alansary et al. 2015). First tracr RNA and crRNA bind targeting sequence. And then Cas9 nucleases recognize sgRNA which is binding form of crRNA and tracrRNA (Schwank, Koo et al. 2013). Therefore, Cas9 nuclease cut forth sequence from PAM Sequence (Fig 3b). I used sgRNA9.1 which is targeting exon9. Cas9 nuclease recognizes PAM sequence and then deletes the sequence in Exon 9. Through this process, I got exon9 specific deleted stm2β knock out cells.(Fig 3c). I could confirm Stim2β knock out cell line through RT-PCR from cDNA. mRNA expression of stim2β was not detected in #13 cell line (Fig 3d). I could validate genomic deletion through genomic DNA PCR as well. Stm2β knock out cells, L1-S2β-KO #13, #14, and #5 showed various deletion phenotypes as shown in Figure 3e-f, respectively.

4. Stim2β knock-out 3T3-L1 cells shows faster adipogenesis than wild type.

To investigate the effect of Stim2 isoforms, I confirmed mRNA level of Stim2α and Stim2β every 12 hours in wild-type. PPARγ2 increases after 24 hours in wild-type (Fig 4a). Also, mRNA expression level of Stim2α and Stim2β are well expressed in wild-type during adipogenesis(Fig 4b). To investigate the effect of Stim2β isoform on differentiation, I measured mRNA level of Stim2α and Stim2β every 12 hours in L1-S2β-KO #13. Interestingly, mRNA level of PPARγ2 increased after 12 hours in L1-S2β-KO #13 (Fig 4c). mRNA expression level of Stim2α are well expressed while mRNA expression level of Stim2β does not expressed in L1-S2β-KO #13 during adipogenesis(Fig 4d). I checked the role of Stim2β in cell proliferation through MTT assay. I found that there is no proliferation difference in WT, and other two knock-out cell lines, L1-S2β-KO #13 and L1-S2β-KO #14 (Fig 4e). I also checked whether Stim2β is involved in differentiation through Oil red O staining. As a result, knock-out cells which are L1-S2β-KO #13 and in L1-S2β-KO #14 showed more accumulated lipid droplet and faster differentiation than WT (Fig 4f). The extent of differentiation was quantified by staining lipid with Oil Red O (Fig 4g).

Figure 1. Differentiation of NIH 3T3-L1 cells into adipocytes.

a.



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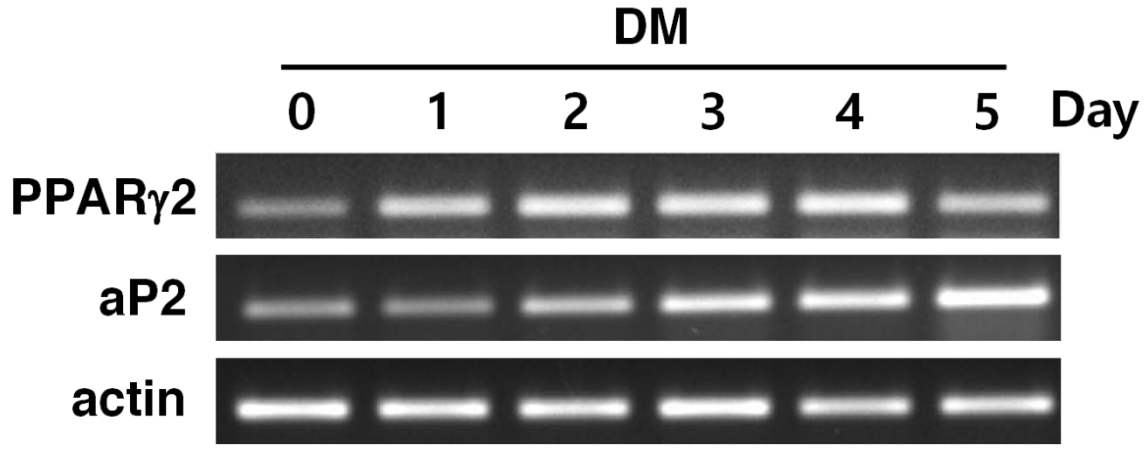


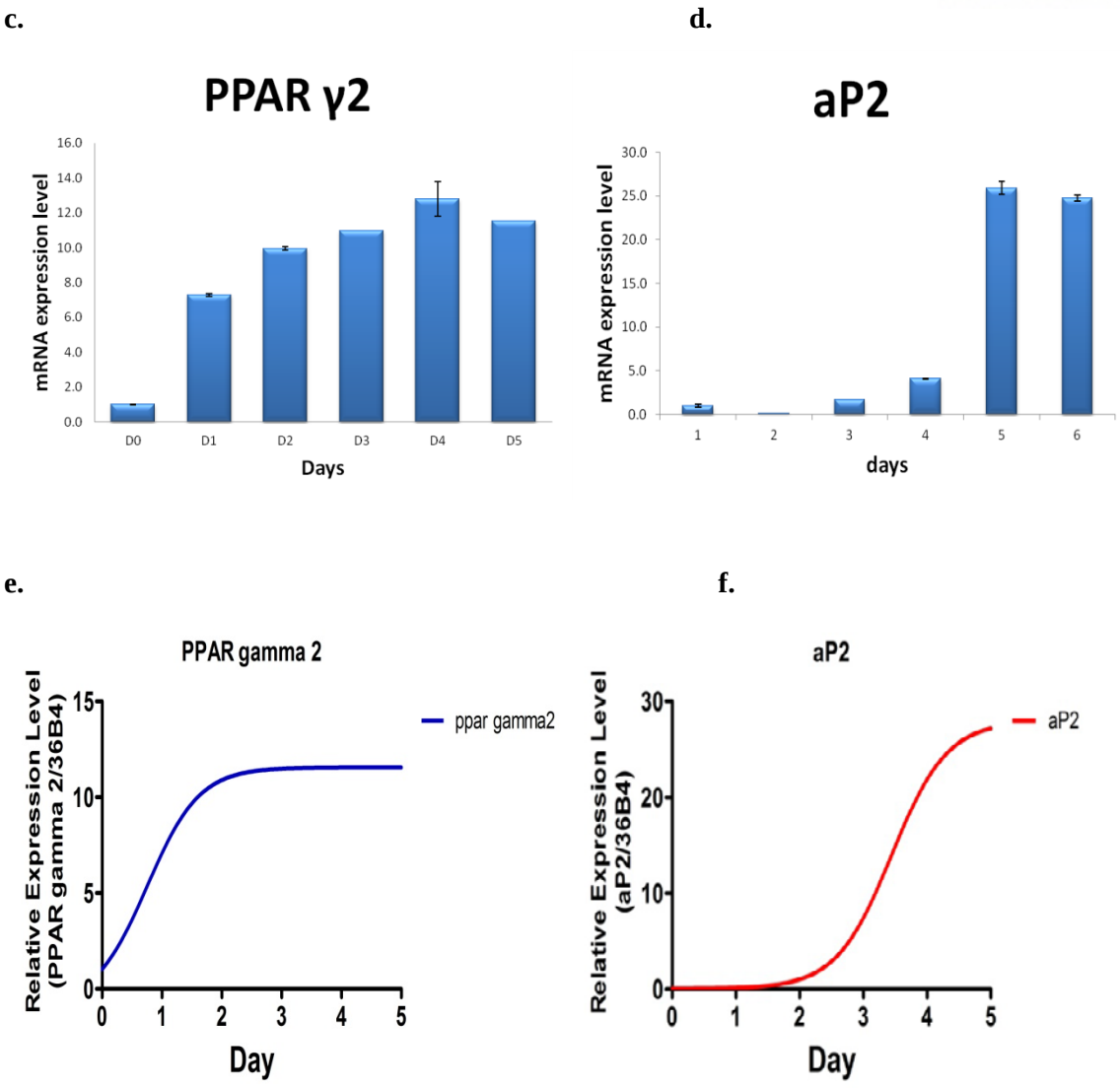
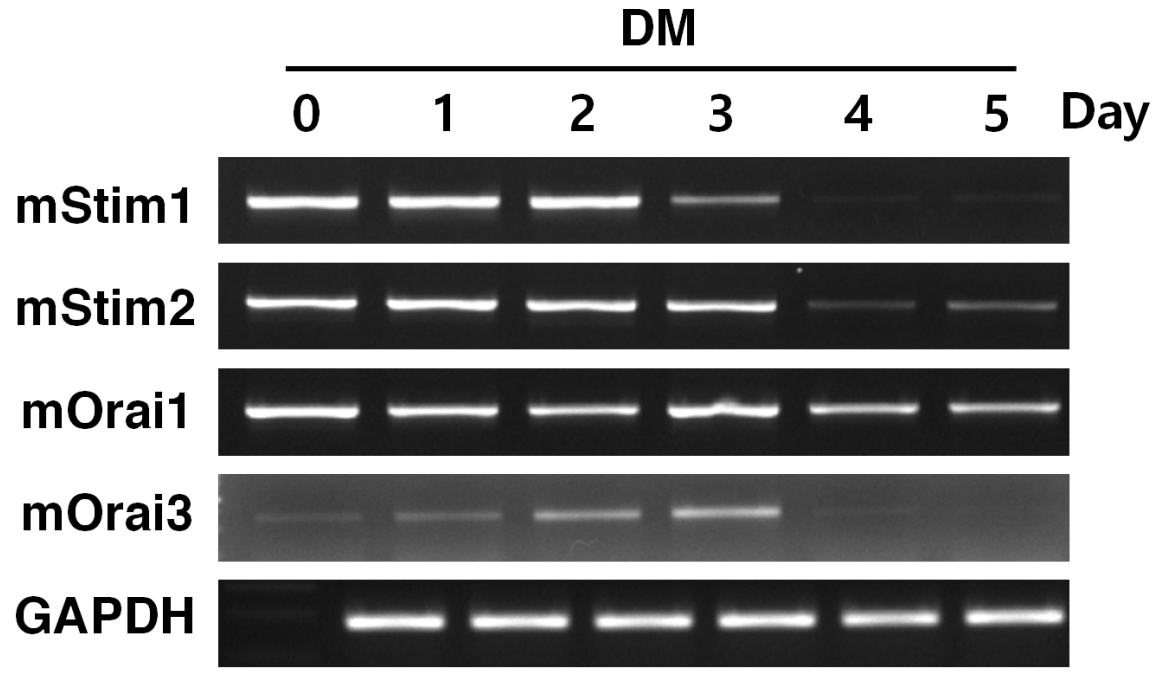


Figure 1. 3T3-L1 cell is differentiated into adipocyte by treating MDI.

a) DIC micrographs of undifferentiated 3T3-L1 cells at Day 0 and induced 3T3-L1 cells on day 1, 3, and 5. b) Adipogenesis marker genes expression by RT-PCR during adipocyte induction. c,d) qRT-PCR gene analysis of adipogenesis marker genes expression, PPARγ2 (c) and aP2 (d). data are the mean ± s.d. of two (three) independent experiments by triplicate (n=3). e.f) Adipogenesis marker genes expression, PPARγ2 (c) and aP2 (d) were normalized by 36B4.

Figure 2. Expression of Stim and Orai isoforms during adipogenesis of 3T3-L1 cells.

a.



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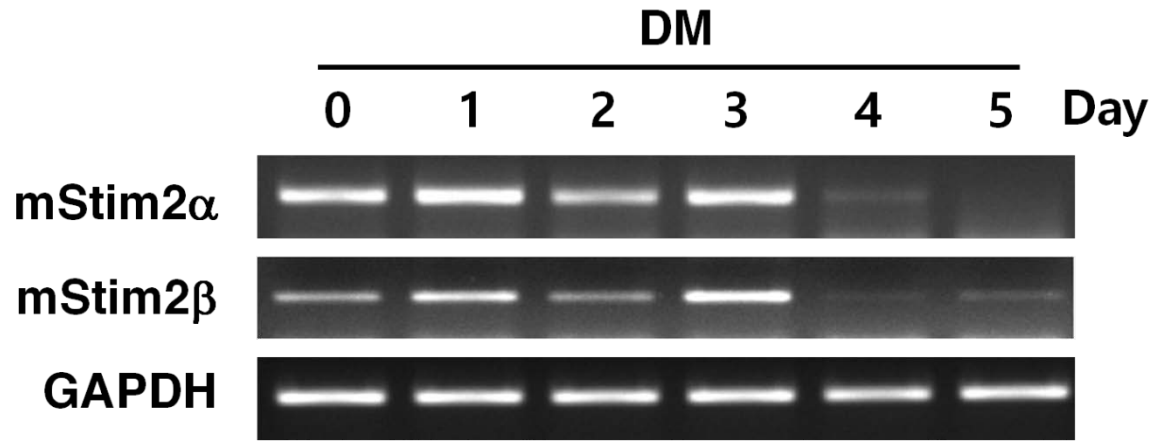


c.

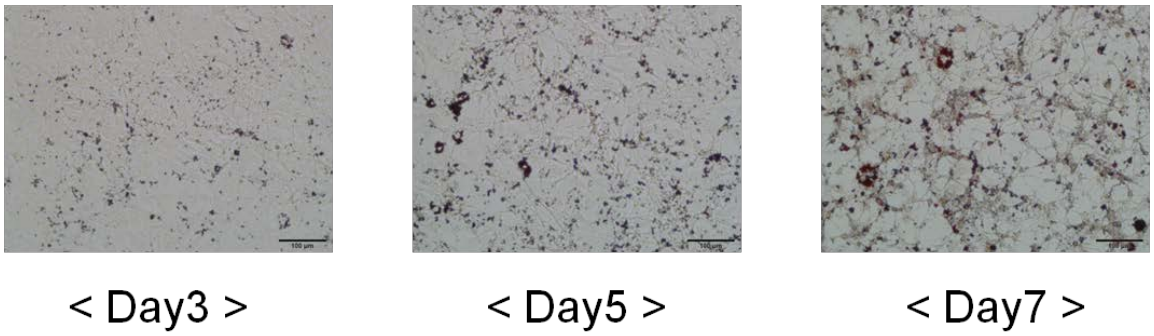
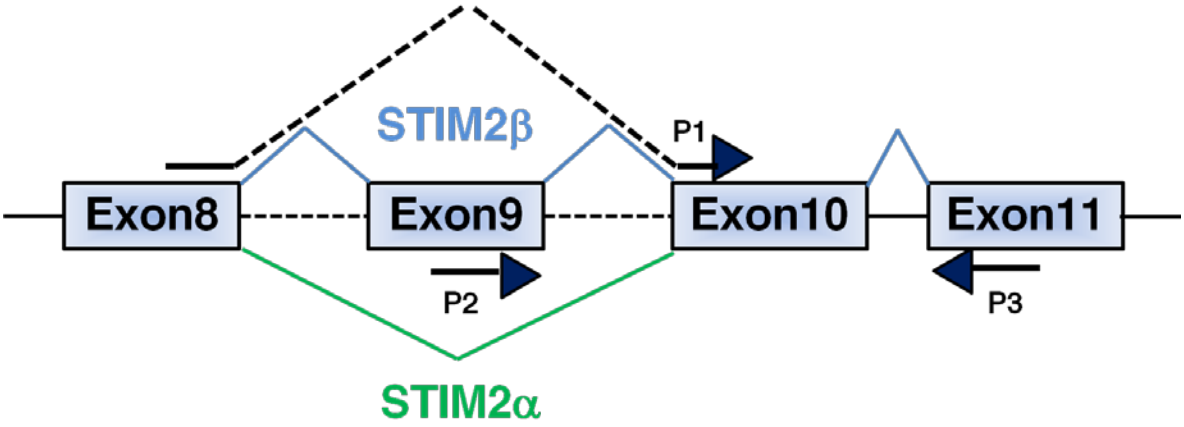


Figure 2. Expression of Stim and Orai isoforms during adipogenesis of 3T3-L1 cells. a) mRNA expression level of SOC components, Stim1, Stim2, Orai1 and Orai3, is detected by semi quantitative PCR during adipogenesis of 3T3-L1 cell at different time points. b) mRNA expression level of Stim2 splicing variants, STIM2α and STIM2β are detected by semi quantitative PCR during adipogenesis of 3T3-L1 cell at different time points. c) 3T3-L1 cells were subjected to Oil Red O staining for the visualization of lipid accumulation at different days of differentiation induced by MDI and following insulin treatment and degree of stained lipid droplets was measured by spectrophotometer. The scale bar indicates 10um.

Figure 3. Generation of stim2β knock-out L1 cell using CRISPR system.

a.



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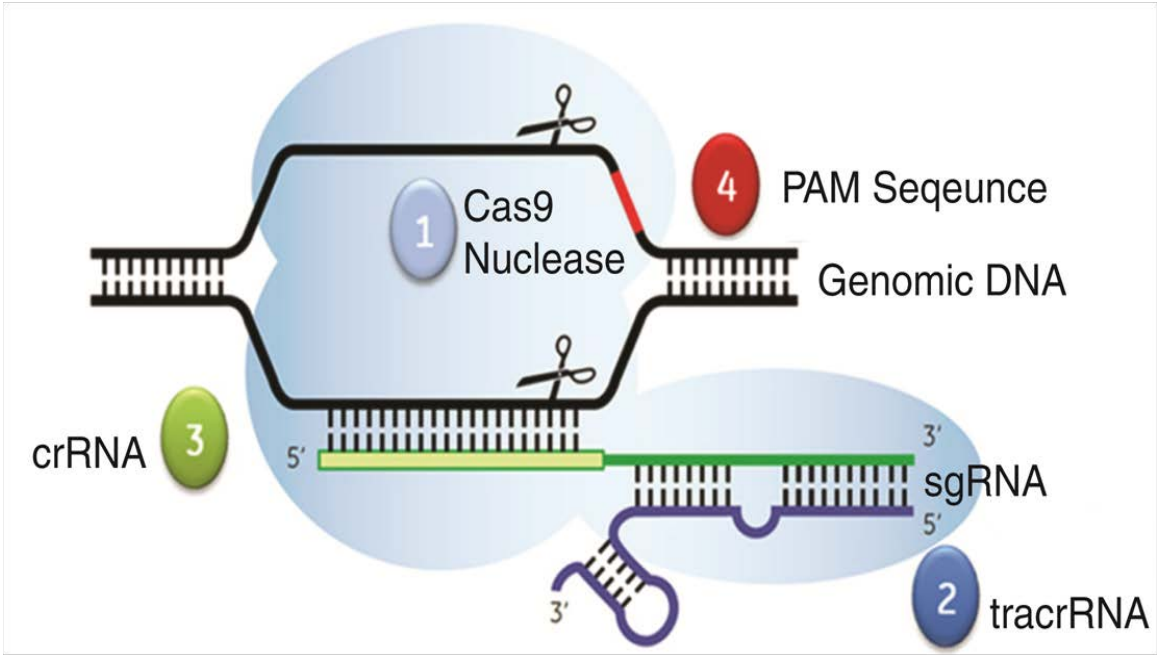
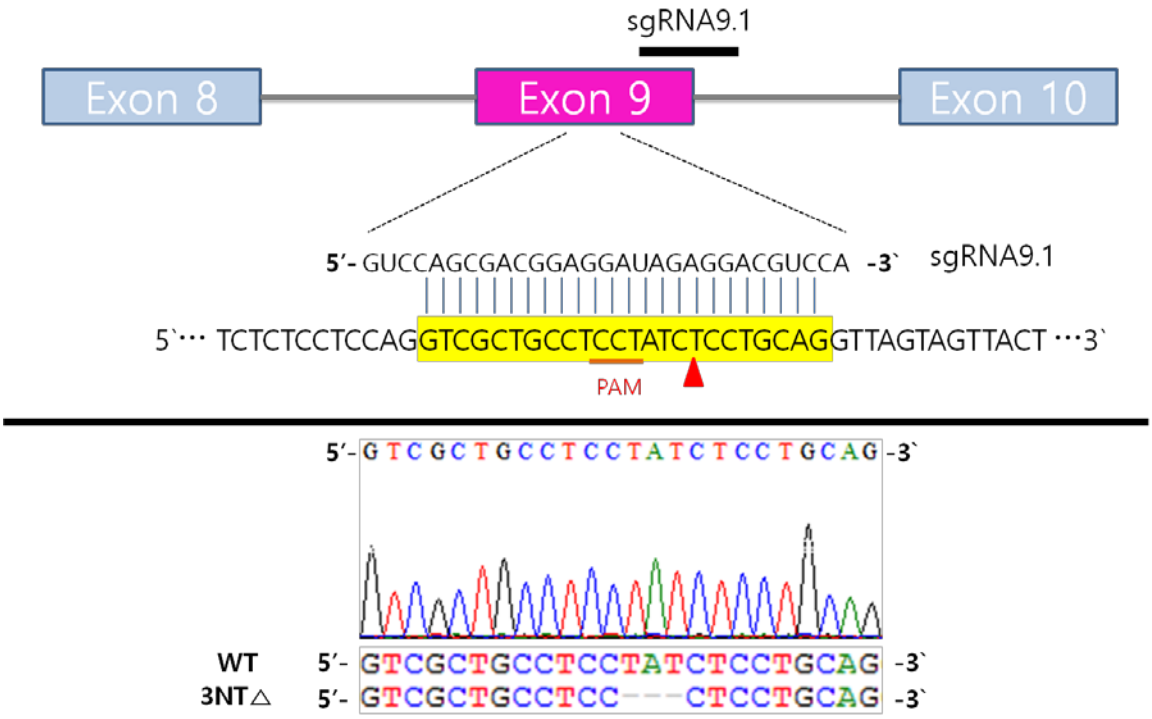


① Cas9 nuclease
② trans-activating crRNA
③ crRNA
④ PAM sequence

c.



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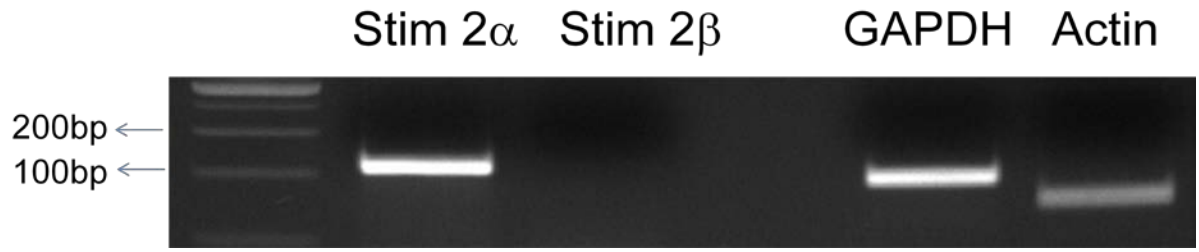

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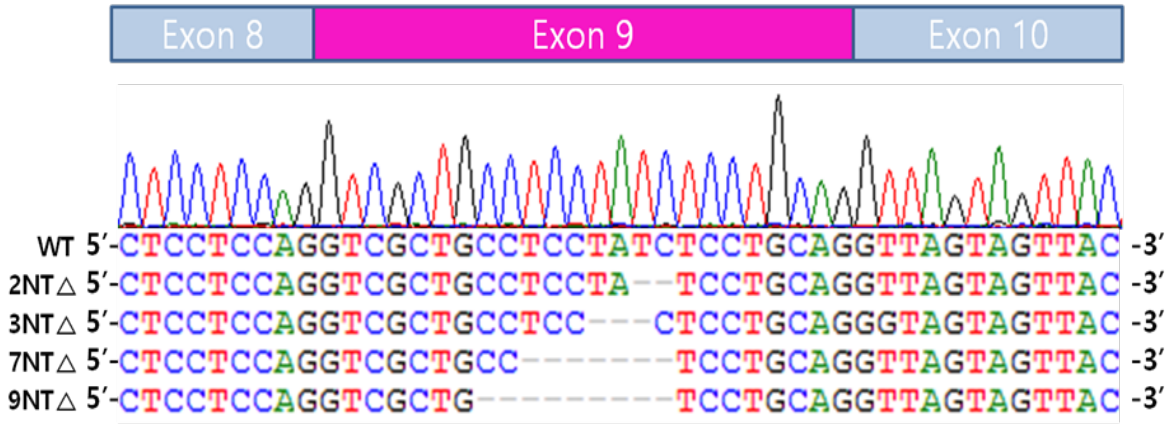

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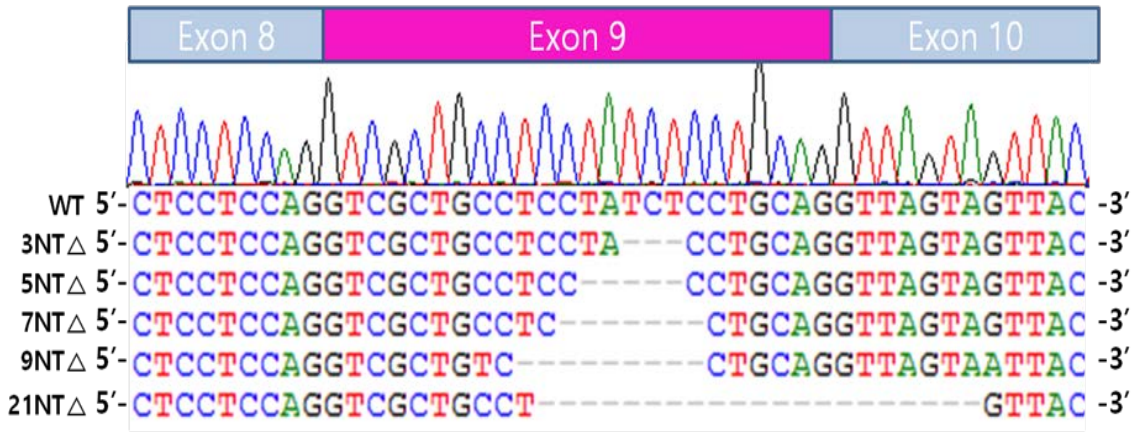

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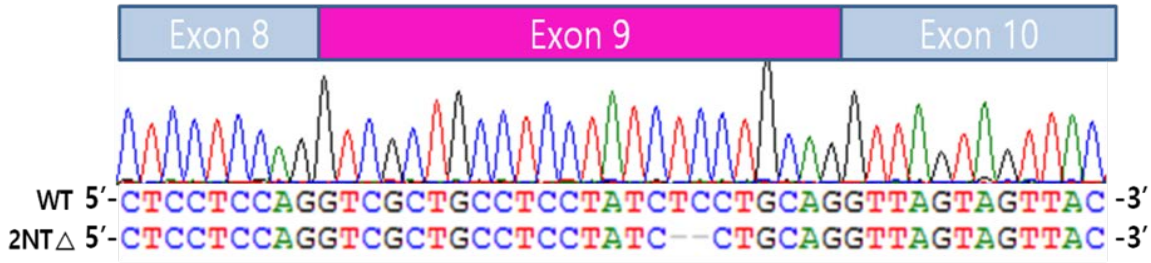

Figure 3. Generation of stim2β knock-out L1 cell using CRISPR system. a) Genomic schematic of alternative splicing of stim2 genomic locus and splicing variant PCR primers are shown. The different primer set 1 (P1 and P3) and set 2 (P2 and P3) were used to amplify for the splicing variant stm2α and stim2β, respectively. b) CRISPR components, which are Cas9 nuclease, crRNA and tracrRNA, work to cut genomic DNA. Assembly of crRNA and tracrRNA is called sgRNA. c) STIM2β knock out is created by targeting indel mutation in Exon 9. Non-Homologous End Jouing (NHEJ) is occurred by CRISPR system. d) PCR analysis shows knock out of STIM2β while STIM2β is well produced by proper splicing mechanism. e) L1-S2β-KO #13 cell line which is edited genomic DNA coded STIM2. L1-S2β-KO #13 cell PCR product of STIM2 gene in genomic DNA is inserted into vector and sequenced. f) L1-S2β-KO #14 which is edited genomic DNA coded STIM2. L1-S2β-KO #14 PCR product of STIM2 gene in genomic DNA is inserted into vector and sequenced. g) L1-S2β-KO #5 which is edited genomic DNA coded STIM2. L1-S2β-KO #5 PCR product of STIM2 gene in genomic DNA is inserted into vector and sequenced.

Figure 4. Stim 2β knock-out 3T3-L1 cells shows faster adipogenesis than wild type.

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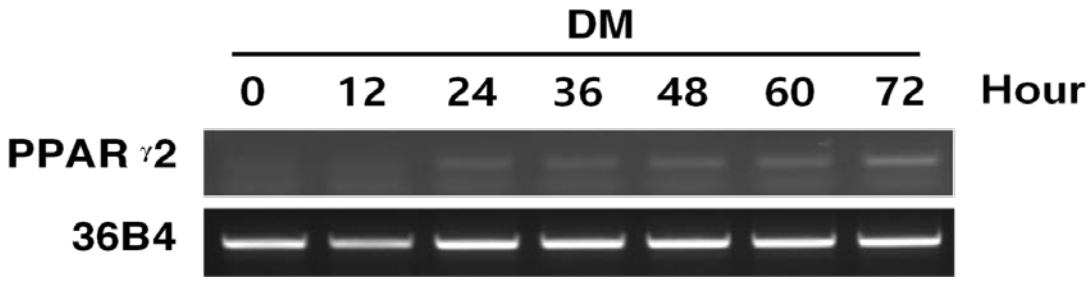

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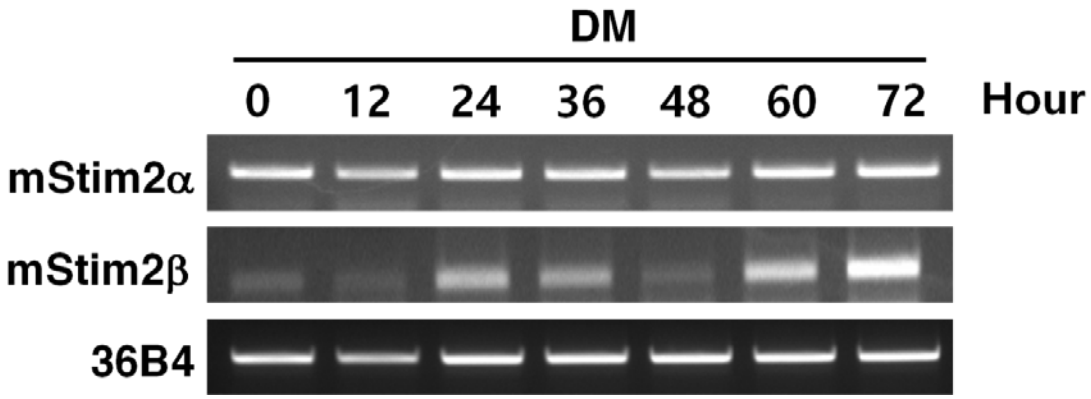

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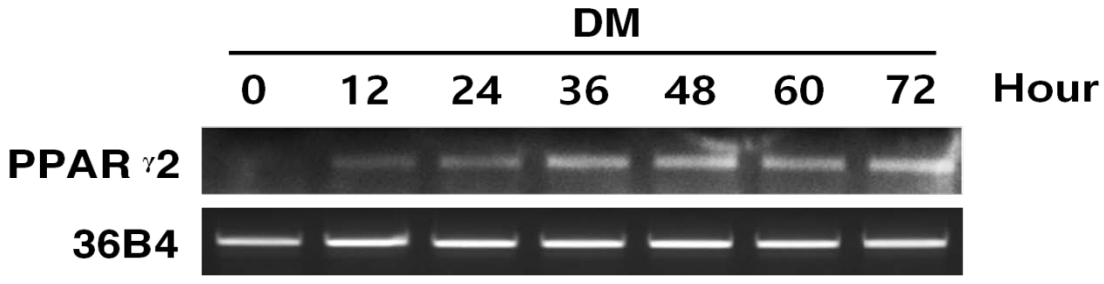

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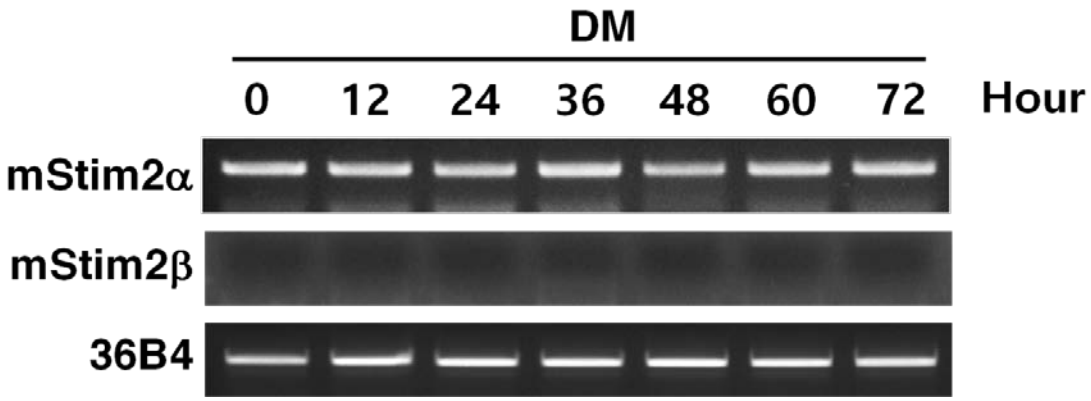

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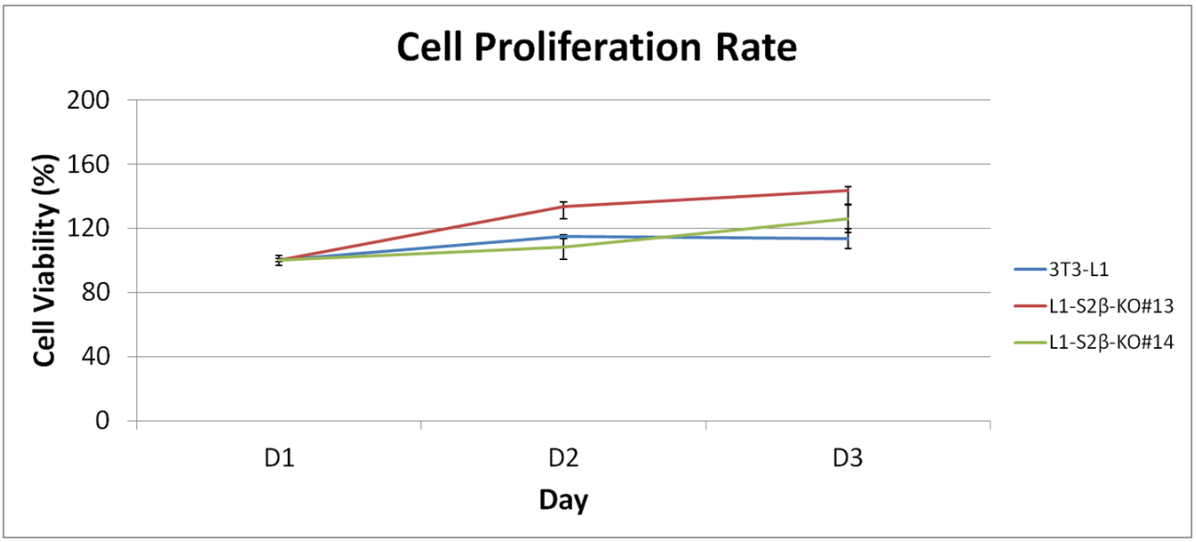

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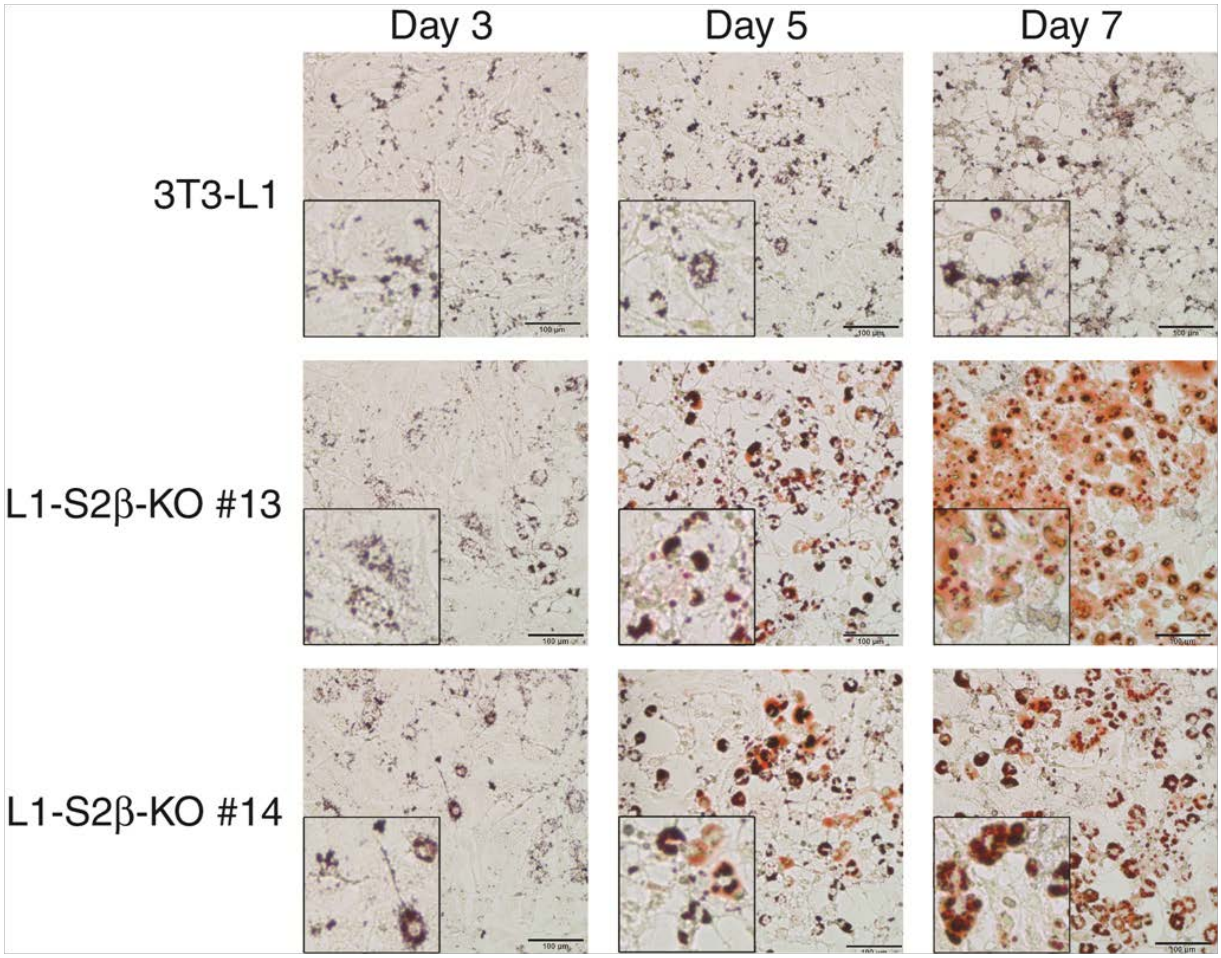

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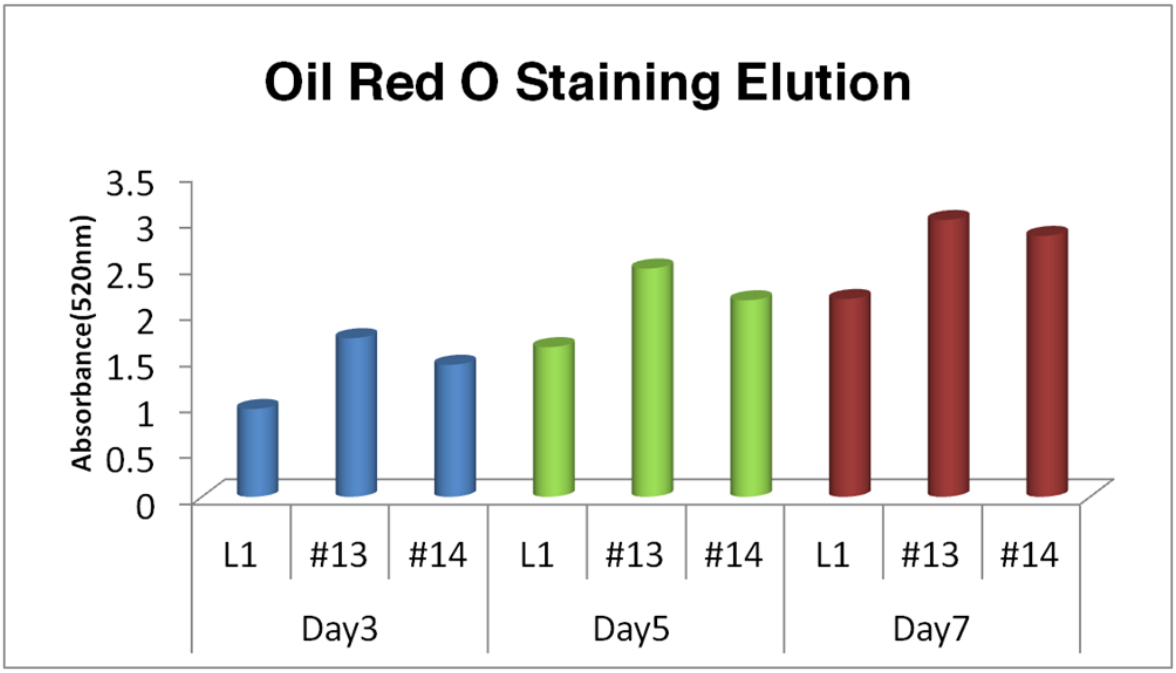

Figure 4. Stim 2β knock-out 3T3-L1 cells shows faster adipogenesis than wild type.

a) mRNA expression level of PPARγ2 is detected by semi quantitative PCR during adipogenesis of wild type 3T3-L1 cell at different indicated time points. mRNA expression of PPARγ2 is increased after 24 hour of MDI induction. b) mRNA expression level of STIM2α and STIM2β is detected by semi quantitative during adipogenesis of wild type 3T3-L1 cell at different indicated time points. Expression level of STIM2α and STIM2β mRNA in 3T3-L1 wild type shows biphasic expression pattern during adipogenesis at 24 and 72 hours after MDI induction. c) mRNA expression level of PPARγ2 is detected by semi quantitative PCR during adipogenesis of Stim2β knockout cell 3T3-L1 cell (L1-S2β-KO #13) at different time points. mRNA expression of PPARγ2 is increased after 12 hour of MDI induction. d) mRNA expression level of STIM2α and STIM2β is detected by semi quantitative during adipogenesis in L1-S2β-KO #13 at different indicated time points. e) MTT proliferation assay was carried out to determine cellular proliferation in WT, 3T3-L1, L1-S2β-KO #13, and L1-S2β-KO #14 seeded 25000 cells per well in 96 well plate. f) Oil Red O staining of wild type 3T3-L1 cells, L1-S2β-KO #13 and L1-S2β-KO #14 for 3 to 7 days in differentiation media containing MDI (1uM dexamethasone, 0.5mM IBMX, and 10ug/ml Insulin). Scale bar indicates 100um. g) Degree of adipogenesis was determined by spectrophotometric quantitation of Oil Red O staining of wild type 3T3-L1 cells, L1-S2β-KO #13 and L1-S2β-KO #14 at indicated time points.

Chapter 2.

Results

Part 2. STIM2β is upregulated by insulin signaling.

1. STIM2α and STIM2β are involved in adipogenesis of 3T3-L1 cells.

I found oscillation pattern of Stim2α and Stim2β expression during adipogenesis induction. When 3T3-L1 becomes confluent state, I treated MDI at day0. After 2 days, I changed media to insulin media at day2. And then after 2 day, I changed media to insulin media at day4 again (Zebisch, Voigt et al. 2012). Stim2α and Stim2β expression were increased after 24 hour from insulin treatment (Fig 1a). qRT-PCR result of Stim2α and Stim2β also clearly showed oscillation pattern (Fig 1b,c). qRT-PCR result is the relative expression level of Stim2α and Stim2βwhich is divided with the mRNA expression level of 36B4, a housekeeping gene. Quantified results are also in agreement with this experiment (Fig 1d.e).

2. Insulin enhances the expression of Stim2α and Stim2β.

To check the effect of differentiation cocktail in gene expression of Stim2α and Stim2β, I treated MDI or insulin to NIH 3T3-L1 cell at day 1. mRNA level of Stim2α and Stim2β was more increased in treatment of MDI or insulin than control at day2 (Fig 2a). Furthermore, SOC components also were affected by MDI or insulin. At the result, I could see various patterns of each SOC components (Fig 2b). To confirm the oscillation pattern by MDI or insulin, I treated MDI or insulin at day0 in NIH 3T3-L1 cell. mRNA level of Stim2α and Stim2β was increased at day1 which is the time after 24 hours of insulin treatment. However mRNA level of Stim2α and Stim2β was decreased at day2 (Fig 2c). I wanted to know which factor increases Stim2 isoform expression. So I treated DEX and IBMX which are components of differentiation cocktail. At the result, I confirmed that stim2 isoform is not affected by DEX and IBMX. However, after treatment of insulin, Stim2 isoform was increased. Therefore I found that insulin affects to Stim2 isoform expression (Fig 2d). To know whether glucose or insulin affects to mRNA expression of SOC components, I treated 0mM glucose without insulin and 5mM and 25mM of glucose in the absence and presence of insulin (Daskoulidou, Zeng et al. 2015). Each SOC components showed various patterns of expression change in various conditions (Fig 2e). I wondered how soon Stim2 expression is regulated by insulin. Therefore, I treated insulin to

NIH 3T3-L1 cells in various time points. As a result, I found that Stim2 isoform expression was increased after 9 hour of insulin treatment (Fig 2f). Next, to check whether glucose regulate Stim2 isoform expression, I treated different amount of glucose in the absence and presence of insulin (Daskoulidou, Zeng et al. 2015). It seems that there is a little effect of glucose. Therefore, Stim2 isoform expression is mainly regulated by insulin rather than glucose(Fig 2g).

Figure 1. STIM2α and STIM2β are involved in adipogenesis of 3T3-L1 cells.

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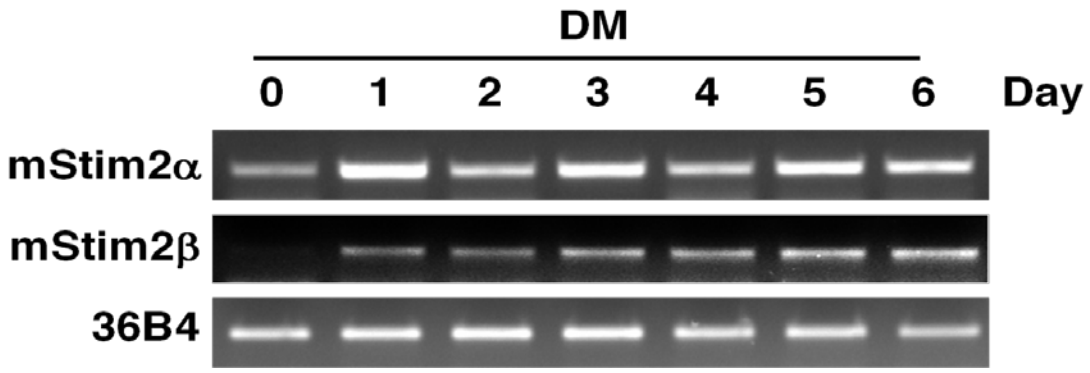

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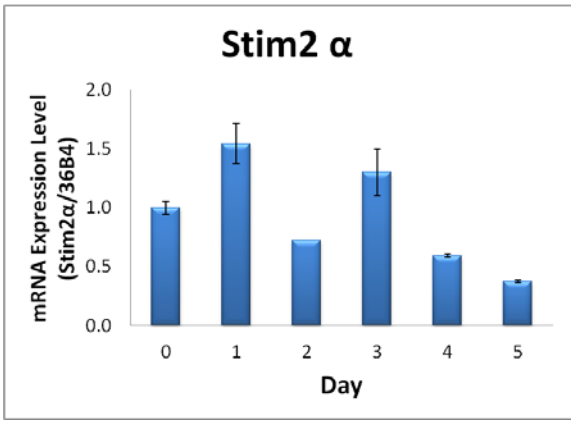

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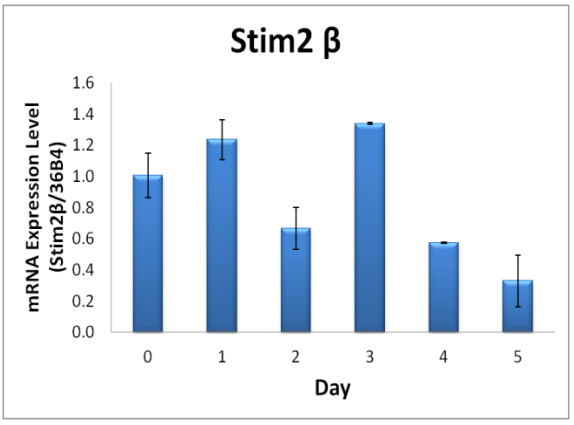

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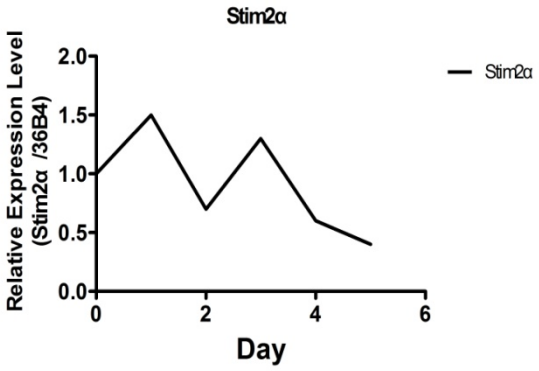

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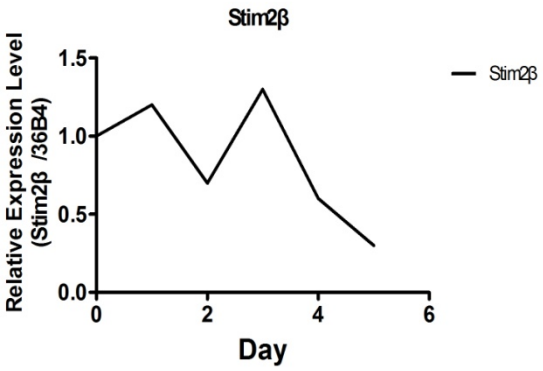

Figure 1. STIM2α and STIM2β are involved in adipogenesis of 3T3-L1 cells. a) Semi quantitative PCR result of STIM2α, STIM2β gene which are splicing variants during adipogenesis. MDI induced in Day 0 and post insulin treatment is applied at Days 2, 4. 36B4 is housekeeping gene. b) mRNA expression level of STIM2α during adipogenesis is detected via qRT-PCR. c) mRNA expression level of STIM2β during adipogenesis is detected via qRT-PCR. d) Relative expression level of STIM2α mRNA shows oscillation pattern which has peak in days 1 and 3 during adipocyte differentiation. e) Relative expression level of STIM2β mRNA shows oscillation pattern which has peak in days 1 and 3 during adipocyte differentiation.

Figure 2. Insulin enhances the expression of Stim2α and Stim2β

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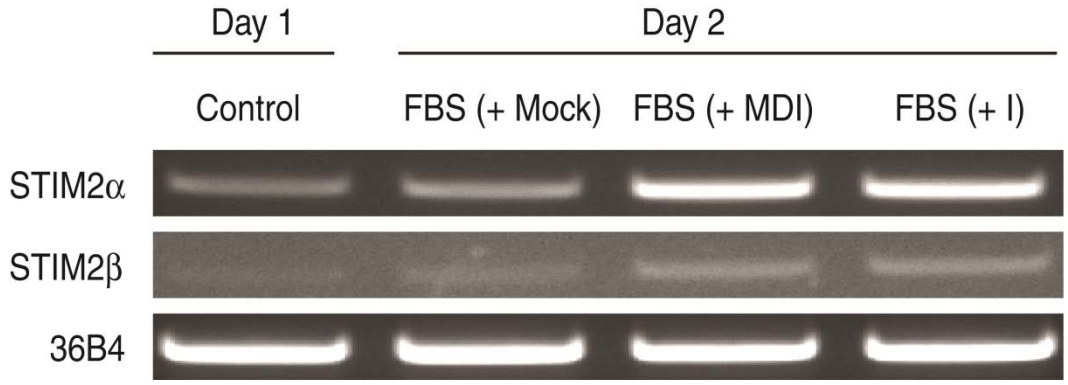

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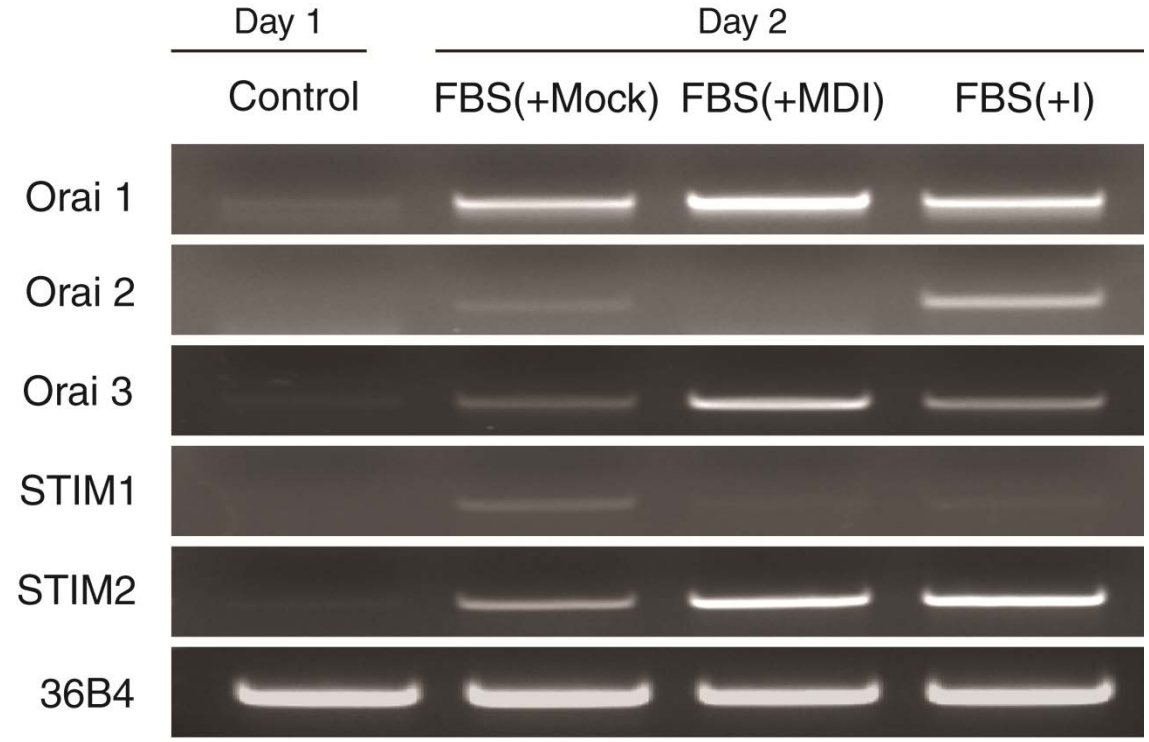

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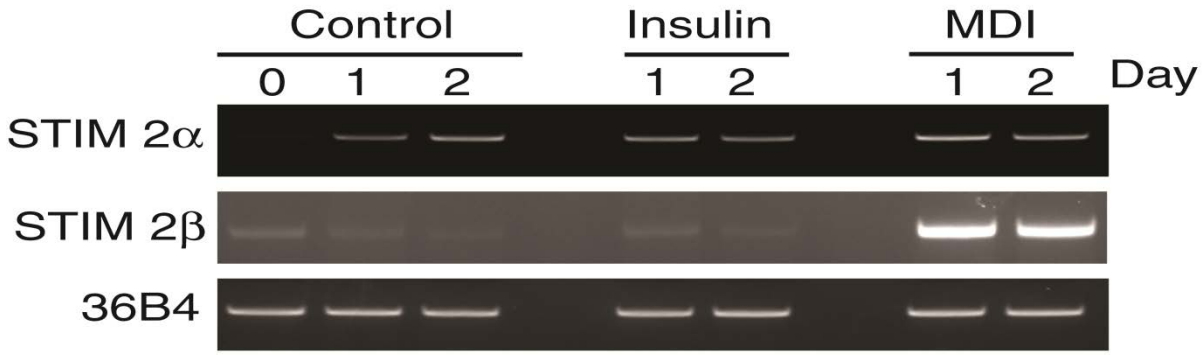

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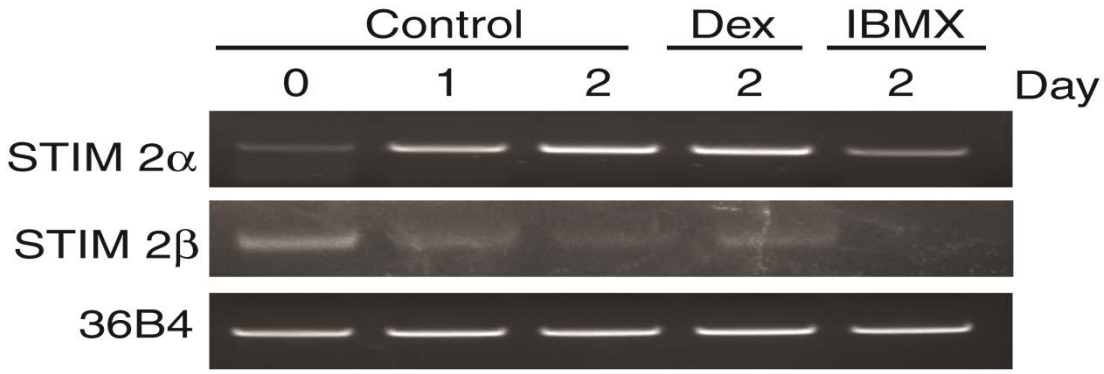

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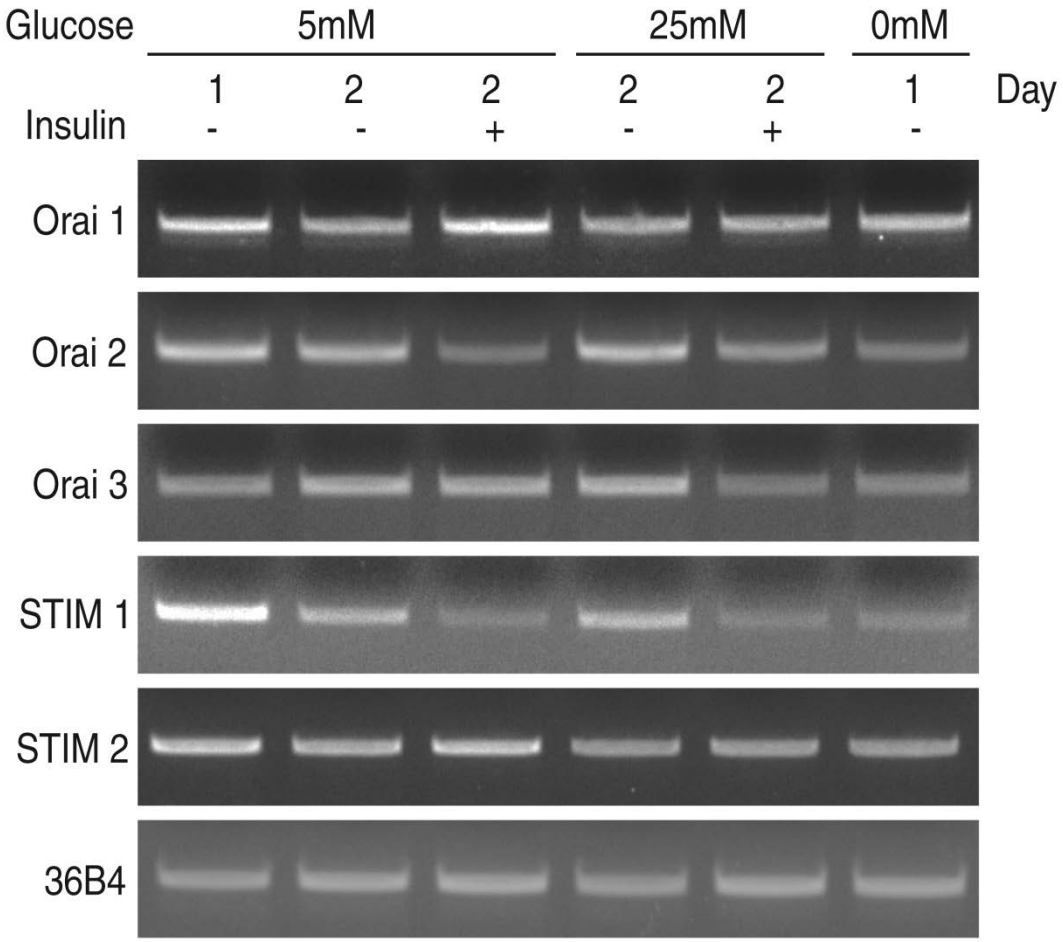

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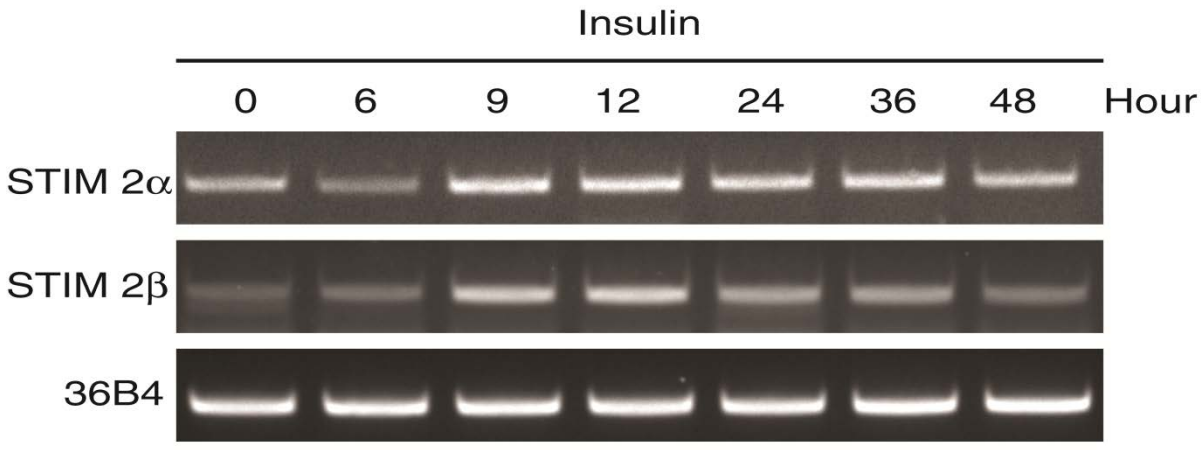

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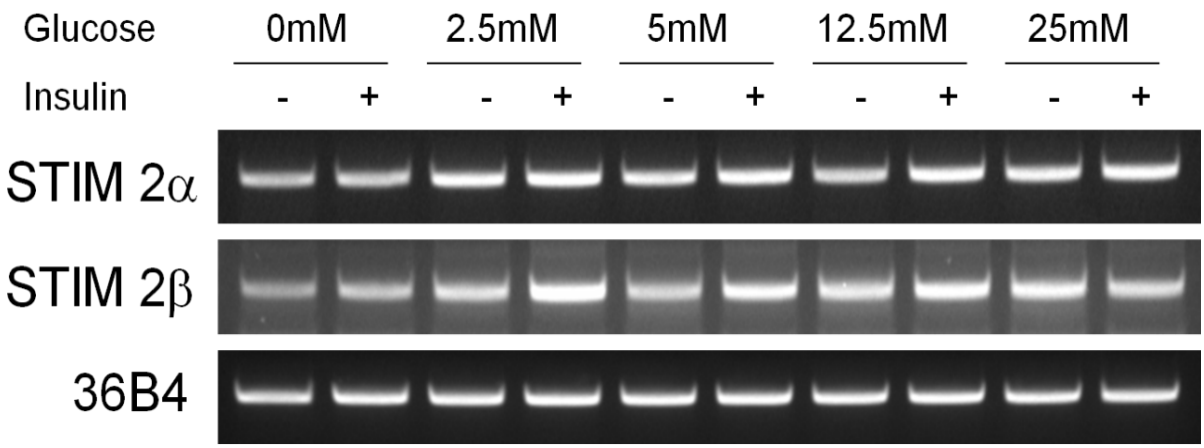

Figure2. Insulin enhances the expression of Stim 2α and Stim 2β a) mRNA level of STIM2α and STIM2β at day 2 are increased after inducing insulin and MDI at day 1. Control is 3T3-L1 cell in BCS media. 36B4 is housekeeping gene. b) mRNA level of SOC component such as Orai 1,2,3 and STIM 1,2 shows various pattern after inducing insulin and MDI at day 1. c) Expression level of STIM2α and STIM2β mRNA is increased by insulin after 24 hours inducing insulin or MDI at day 0. d) mRNA level of STIM2α and STIM2β are not affected by dexamethaxon and isobutyl methyl xanthine which are differentiation components of adipogenesis. e) mRNA expression level of SOC components shows various pattern in 0mM, 5mM, 25Mm Glucose media. Insulin is treated at day 1. f) mRNA level of STIM2α and STIM2β is increased after 9 hours from insulin induction at 0 hours. g) Expression level of STIM2α and STIM2β mRNA is affected by insulin rather than Glucose level of media 0mM, 2.5mM, 5mM, 12.5mM, 25mM.

Discussion

The STIM proteins are well established for two major functions in ER : sensing calcium ion concentration in ER and activating Store-Operated Calcium Channel by binding with Orai protein. Recent studies published that alternative splicing variant of STIM2 which is STIM2β can inhibit the SOC function by regulating STIM1 (Rana, Yen et al. 2015).

In this research, I establish a role of STIM2β in adipogenesis of NIH 3T3-L1 cell. I have shown that STIM2β plays an important role in NIH 3T3-L1 cell adipogenesis not cell proliferation. Besides, I show that STIM2β knock out 3T3-L1 cell has faster lipid droplet formation than wild type. STIM2β knock out cell that is generated by CRISPR system. CRISPR system is originated from the prokaryotic immune system that is operated by Cas9 nuclease, crRNA, tracrRNA and PAM sequence (Hwang, Fu et al. 2013). Also, I found that STIM2 isoform shows oscillated expression pattern and their mRNA expression level is regulated by insulin rather than glucose. DEX and IMBX which are the components of differentiation cocktail do not affect to the mRNA expression of STIM2 isoform. Thus, STIM2β is important for adipogenesis and insulin regulates STIM2β expression level. Therefore, insulin regulates gene expression of STIM2 isoform. Then, calcium imbalance mediated by STIM2β which is calcium modulator affects early differentiation in NIH 3T3-L1 cell.

I found that mRNA expression level of STIM2 isoform is regulated by insulin rather than DEX, IMBX or glucose. Also I establish that STIM2 isoform has oscillated expression pattern. Some study shows that high glucose level is involved in SOC components expression level (Daskoulidou, Zeng et al. 2015) . Glucose uptake of cell is regulated by GLUT4 channel in membrane (Watanabe, Hisatake et al. 2015). However, in this research, regulation of STIM2α and STIM2β expression level is more affected by insulin than glucose concentration. Insulin signaling is mediated by Insulin Receptor Substances (IRS), Akt and Phosphatidyl Inositol 3-Kinase (PI3K) (Gao, Zhang et al. 2015). However, the exact mechanism by which the regulation of STIM2 isoform mRNA expression is upregulated after 9 hours from insulin treatment remains elusive. One possible mechanism is that there is IRE binding sequence in STIM2 promoter. Therefore, I can hypothesis that insulin might control the STIM2 isoform expression level through by the IRE promoter site of STIM2.

Thus, for establishing the exact mechanism, I need some further experiments. To confirm the calcium ion concentration change in 3T3-L1 cell and STIM2β knock out cell, I need to measure intracellular calcium ion level by Fura-2 calcium imaging. Besides, RNAseq can find the calcium ion dependent transcription factors for regulating adipogenesis. Also, with biochemical approach such as immunocytochemisty and immunoprecipitation, I can confirm the change of SOC components expression in protein level. Last, it is important to check the adipogenesis in 3T3-L1 by STIM2β rescue experiment.

There are many attempts for regulation of adipogenesis and obesity treatment. Many studies show that SOC components are related with the adipogenesis (Graham, Black et al. 2009). Especially, through this study, I found that STIM2β which is mediated by insulin might decrease adipogenesis. Thus, downregulation of adipogenesis by STIM2β may provide the potential therapeutic approach for the obesity and related metabolic diseases.

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